(54) Title: TRANSGENIC ANIMALS AS MODELS FOR NEURODEGENERATIVE DISEASE

(57) Abstract: There is provided a nucleic acid vector comprising: (a) a nucleic acid sequence encoding a human Tau protein; (b) a sequence capable of directing expression of said human Tau protein in the nervous system of a non-human animal; and (c) a targeting sequence which facilitates integration of said vector into the genome of said animal so as to prevent expression of equivalent Tau protein or a related or equivalent protein from said animal in favour of said human Tau protein. A further aspect provides a nucleic acid vector comprising: (a) a nucleic acid sequence encoding a human protein capable of modulating human Tau protein; (b) a sequence capable of directing expression of said protein in the nervous system of said animal; and (c) a targeting sequence capable of facilitating integration of said vector into the genome of said animal optionally at a position corresponding to a sequence in said animal encoding an equivalent of said human protein so as to prevent expression of said equivalent sequence in favour of said human protein capable of modulating human Tau protein. The vectors are used to make a transgenic non-human animal by (a) introducing into an embryo cell of said animal one or more of a nucleic acid vector as described above; (b) introducing the embryo from step (a) into a female animal; (c) sustaining the female in step (b) until such time as the embryo has sufficiently developed and is borne from the female; and (d) sustaining the transgenic animal.

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TRANSGENIC ANIMALS AS MODELS
FOR NEURODEGENERATIVE DISEASE

5   The present invention relates to cell and animal
      models for a disease condition and in particular to an
      animal model which can function as a model for
      neurodegenerative diseases, such as Alzheimers.

      Alzheimers disease is a neurodegenerative disorder
10   which is the most prevalent form of senile dementia,
      with approximately 5% of individuals of 65 and 20% of
      those over so being afflicted. The disease is
      characterised by the appearance of two principal
      lesions within the brain termed neurofibrillary
15   tangles and senile plaques.

      Neurofibrillary tangles are intracellular inclusion
      bodies which comprise filamentous aggregates of paired
      helical filaments (PHF). The principal component of
20   PHF has been shown to be Tau, a microtubule associated
      protein involved in stabilising the cytoskeleton and
      in determining neuronal shape. Tau is a
      phosphoprotein and aberrant hyper phosphorylation of
      Tau appears to represent one mechanism for its
25   aggregation into PHF. Biochemical analysis and
      structural prediction of the phosphorylation sites of
      human protein Tau of paired helical filaments (PHF) in
      brain of Alzheimer's disease (AD) patients revealed
      that many sites consist of serine or threonine
30   residues followed by a proline residue, focussing
      attention on proline dependent kinases (Wood et al.,
      1986; Wischik et al., 1988; Brion et al., 1991;
      Hasegawa et al., 1992; Pollanen et al., 1997).

35   Further neurodegenerative disorders mediated by Tau
      positive filamentous lesions include, FTDP-17 (Fronto-

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temporal dementia associated with Parkinson's
disease), Cortico-basal degeneration, progressive
supranuclear palsy, multiple system atrophy , Pick's
disease, Dementia Pugilistica, Dementia with tangles
only, dementia with tangles and calcification, Down
syndrome, Myotonic dystrophy, Niemann Pick's disease
type C, Parkinsonism-dementia complex of Guam,
Postencephalic Parkinsonism, Prion diseases with
tangles, subacute sclerosing panencephalitis.

Despite the data currently available, convincing
evidence in addition to a suitable animal model
demonstrating and exhibiting the phosphorylation of
protein Tau by human kinases *in vivo*, is lacking.

The present invention is therefore directed to
providing an animal model of neurodegenerative
diseases, such as Alzheimers and which model may be
utilised to identify compounds useful in treating or
ameliorating the symptoms of the condition.

In a first aspect the present invention provides a
nucleic acid vector comprising a) a nucleic acid
sequence encoding a human Tau protein; b) a sequence
capable of directing expression of said Tau protein in
the nervous system of said animal; and c) a sequence
which facilitates integration of said vector into the
genome of said animal so as to prevent functional
expression of said animal Tau protein in favour of
said human Tau protein. This construct or vector
thus permits generation of cells of non human animals
which express the human Tau and which are
substantially uncontaminated with endogenous Tau
proteins from the animal or cell. Thus, such a cell
or non-human animal may be particularly useful as a
model to monitor the function of human Tau proteins
and its potential role in the progression of

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neurodegenerative disorders mediated by Tau protein, such as Alzheimer's disease.

In one embodiment of the invention, the sequence which
5   facilitates integration of the vector into the genome comprises a sequence of nucleotides which exhibits a sufficient degree of homology with the Tau sequence of the animal or the flanking regions thereof, to permit homologous recombination and subsequent insertion of
10 the vector into the genome of said animal at a location which disrupts the coding region and hence expression of the endogenous Tau in said animal in favour of the human Tau protein encoded from the sequence present on said vector. Whilst it will be
15 appreciated by the skilled practitioner that a range of sites upstream, downstream or within the endogenous Tau sequence in the animal genome may be utilised as the site of homologous recombination, it is preferred that the region of homology is selected such that
20 expression of proteins from other gene coding sequences upstream or downstream of the endogenous Tau sequence are not affected. As discussed in more detail in the example below, the vector of the invention may be targeted to, for example, the
25 corresponding Tau sequence of a mouse by the inclusion of a NcoI restriction fragment suitable for insertion of the vector into the unique NcoI site in exon 1 of the Tau sequence in the mouse genome, although as aforementioned a range of appropriate regions of
30 homology to sites upstream or downstream of said Tau sequence may be used.

An expression vector according to the invention includes a vector having a nucleic acid according to
35 the invention operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term

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"operably linked" refers to a juxta position wherein
the components described are in a relationship
permitting them to function in their intended manner.
Such vectors may be transformed into a suitable host
cell to provide for expression of a polypeptide
according to the invention. Thus, in a further
aspect, the invention provides a process for preparing
receptors according to the invention which comprises
cultivating a host cell, transformed or transfected
with an expression vector as described above under
conditions to provide for expression by the vector of
a coding sequence encoding the receptors, and
recovering the expressed receptors.

The vector according to the invention is termed a
"knock in-knock out" vector by virtue of the fact that
the endogenous Tau protein is prevented from being
expressed in favour of the exogenous DNA sequence.
Preferably, such a vector further comprises a marker
sequence which in one embodiment may comprise the
hygromycin marker gene Pgk-hyg.

The sequence encoding the Tau protein is preferably a
cDNA sequence, and even more preferably encodes one of
the Tau 40 isoforms already known in the art (Goedert
M, Trends Neuroscience 1993 Nov; 16(11): 460-465).
However, although the known sequences encoding human
Tau isoforms may be utilised, mutated Tau sequences
may be used to investigate the role of Tau protein in
the pathology of neurodegenerative disorders in an
animal mediated by Tau protein.

A second aspect of the invention comprises a further
nucleic acid vector comprising (a) a nucleic acid
sequence encoding a protein capable of modulating a
human Tau protein; (b) a sequence capable of directing
expression of said protein in the cells of said

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animal; and (c) a targeting sequence capable of
facilitating integration of said vector into the
genome of said animal optionally at a position
corresponding to a sequence in said animal equivalent
5   to said protein capable of modulating human Tau
protein, so as to prevent expression of said
equivalent sequence in favour of said protein capable
of modulating human Tau protein.

10   Such a vector when integrated at said equivalent
sequence in the animal genome, in a similar fashion to
the vector described above, permits expression of the
protein capable of modulating Tau protein in favour of
the related or equivalent protein in said animal.
15

The sequence capable of directing expression of said
human Tau protein or the modulator thereof is
preferably a transcriptional control sequence which
can steer expression of the proteins to the nervous
20   system of the non-human animal. Transcriptional
control sequences according to the invention comprise
a suitable promoter and other regulatory regions, such
as enhancer sequences, that can modulate the activity
of the promoter.
25

Regulatory elements required for expression include
promoter sequences to bind RNA polymerase and
transcription initiation sequences for ribosome
binding. For example, a bacterial expression vector
30   may include a promoter such as the lac promoter and
for transcription initiation in the Shine-Dalgarno
sequence and the start codon AUG. Similarly, a
eukaryotic expression vector may include a
heterologous or homologous promoter for RNA polymerase
35   II, a downstream polyadenylation signal, the start
codon AUG, and a termination codon for detachment of
the ribosome. Such vectors may be obtained

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commercially or assembled from the sequences described
by methods well known in the art.

A promoter refers to the region of DNA that is
upstream with respect to the direction of
transcription of the transcription initiation site and
which promoter is in a relationship permitting
expression of the relevant proteins according to the
invention.

DNA sequences that drive expression to neurons are
known. They include both control systems that are
neuron-specific and control systems that are more or
less promiscuous but that induce high levels of
expression in neurons. Depending on the nature of the
construct used in the production of the transgenic
animal and, in particular, the control elements, the
desired proteins may be expressed in all neurons or
only in restricted subsets of neurons of transgenic
animals. Neuron-specific control systems, that drive
expression to neuronal cell types in general, are
known. They may be derived from genes encoding
neuron-specific proteins. Such systems may be used to
bring about expression of the desired Tau protein
and/or the protein capable of it's modulation, in
neurons.

Preferably the sequence is a promoter which directs
expression of said proteins in the neurons of the
brain or other such cells including astrocytes,
oligodendrocytes microglia or Schwann cells.
Preferably, the promoter is the mouse Thy-1 promoter
which drives expression in mouse central neurons.

The vector according to this aspect of the invention
may, advantageously, be used in combination with the
vector incorporating the sequence encoding the human

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Tau protein described above to transfect a non-human
animal and thus provide a transgenic animal which
serves as a model permitting investigation into the
interactions between the protein capable of modulating
5   human Tau protein and said Tau protein and
identification of potential therapeutic agents capable
of modulating the effects of the phosphorylation of
Tau.

10   Alternatively, a transgenic animal incorporating the
nucleic acid vector encoding said human Tau protein
may be crossed with another transgenic animal
comprising the vector encoding said protein capable of
modulating human Tau protein which may result in
15   offspring which express both of the proteins.

The human sequences introduced into the transgenic
animals may encode those which are known in the art.
Preferably, the human Tau comprises a human Tau
20   isoform already known in the art. Alternatively, the
sequence may have been subject to a mutation, such as
for example, a point mutation which may simulate a
mutation that gives rise to certain genetic diseases.
There are techniques known in the art to mutate a
25   desired genetic region so as to inactivate or alter
function or expression of the protein. Such
techniques include homologous recombination. Methods
for detecting homologous recombinant events include
the polymerase chain reaction or by using marker or
30   reporter genes which are only expressed in the event
of a successful targeted recombinant event. Such
mutated sequences when expressed in a transgenic non-
human animal can advantageously, be used to
investigate their effect on the phenotype of said
35   animal and its role in the progression of
neurodegenerative disease, such as Alzheimers mediated
by Tau protein.

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In a preferred embodiment of the second aspect of the
invention, the protein capable of modulating human Tau
protein is a kinase, and preferably one which is
capable of phosphorylating human Tau protein , such as
5   human GSK-3β kinase, for example. *In vitro* assays
have identified glycogen synthase kinase-3β (GSK-3β)
as one candidate involved in phosphorylation of Tau.
Phosphorylation by GSK-3β of bovine (Ishiguro et al.,
1992a and 1992b) and human protein Tau (Hanger et al.,
10   1992; Mandelkow et al., 1992) in cell-free systems,
resulted in phosphorylation patterns of protein Tau
that resembled those of the protein isolated from PHF
from AD brain (Ishiguro et al., 1993). The *in vitro*
phosphorylation of human recombinant protein Tau by
15   GSK-3β reduced its ability to induce microtubule
nucleation (Utton et al., 1997), while the kinase also
phosphorylated neurofilament proteins on specific
domains (Guan et al., 1991). Further evidence for GSK-
3β as a potential protein Tau and neurofilament kinase
20   has been obtained in transfected cells, wherein both
protein Tau (Lovestone et al., 1994; Anderton et al.,
1995; Lovestone et al., 1996; Lovestone and Reynolds,
1997) and NF-H were identified as substrates. Co-
transfection of GSK-3β with Tau in CHO cells increased
25   its phosphorylation concomitant with loss of prominent
bundles of microtubules (Wagner et al., 1996), while
co-transfection with NF-H in COS cells caused
electrophoretic mobility retardation and the
appearance of phosphate-dependent antibody profiles.
30

The involvement of GSK-3β in the hyperphosphorylation
of Tau, both in cultured neurons and in vivo in brain,
was indirectly supported by the finding that lithium,
as inhibitor of GSK-3β, caused Tau dephosphorylation
35   at the sites recognized by antibodies Tau-1 and PHF-
1,which are two of the major epitopes typically
associated with PHF in AD brain. The physiological

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role of GSK-3β was proposed to be in stabilizing the
neuronal cytoskeleton by controlling phosphorylation
of Tau and neurofilament-H and eventually other
substrates (Takahashi et al., 1994). In addition, GSK-
3β plays a role in the development of the brain of
Xenopus as part of the Wingless signaling pathway in
which the kinase is a negative regulator of
dorsoventral axis formation. In this mechanism,
phosphorylation of β-catenin, mediated by axin or
conductin, controls the degradation of β-catenin by
the ubiquitin-proteasome pathway (Aberle et al., 1997;
Behrens et al. 1998; Ikeda et al., 1998). Thus, the
model is particularly useful to investigate the
molecular basis for Alzheimers and other
neurodegenerative disorders mediated by Tau protein
and to investigate compounds which may alleviate the
symptoms of the disease.

The vectors may be transformed into a suitable host
cell which is preferably eukaryotic, which may itself
be used to transform a non-human animal. Thus, in a
further aspect the invention provides a process for
preparing human Tau protein or a protein capable of
modulating Tau protein, comprising cultivating a host
cell transformed or transfected with a vector
according to the invention, under conditions to
provide for expression by the vector of said proteins,
and recovering the expressed proteins. Preferably,
the host cell is a non-human animal cell, and even
more preferably, an embryonic cell of a non-human
animal.

Incorporation of the nucleic acid sequences into the
vector according to the invention for subsequent
transformation and integration into the genome of said
host cell or non-human animal is carried out by
procedures well known to those skilled in the art as

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provided in Sambrook *et al.*, Molecular Cloning, A
Laboratory Manual, Cold Spring Harbour Laboratory
Press. The vector may be introduced by transfection
or other suitable techniques such as electroporation.

5   In the present invention, the incorporation of the
exogenous DNA into the genome of the animal is
accomplished by electroporation of the vector in
embryonic stem cells. The cells that have the
exogenous DNA incorporated into their genome by

10   homologous recombination may subsequently be injected
into blastocysts for generation of the transgenic
animals with the desired phenotype. Successfully
transformed cells which contain the vector according
to the invention may be identified by well known

15   techniques, such as lysing the cells and examining the
DNA by, for example, Southern blotting or using the
polymerase chain reaction.

The vectors may be, for example, plasmid, virus,
20   cosmid or phage vectors, and may contain one or more
selectable markers such as the hygromycin marker gene
Pgk-hyg.

The present invention also advantageously provides
25   nucleic acid sequences of at least approximately 10
contiguous nucleotides of a nucleic acid according to
the invention and preferably from 10 to 50 nucleotides
even more preferably, the nucleic acid sequence
comprise the sequences illustrated in Table 1. These
30   sequences may, advantageously be used as probes or
primers to initiate replication, or the like. Such
nucleic acid sequences may be produced according to
techniques well known in the art, such as by
recombinant or synthetic means. They may also be used
35   in diagnostic kits or the like for detecting the
presence of a nucleic acid according to the invention.
These tests generally comprise contacting the probe

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with the sample under hybridising conditions and
detecting for the presence of any duplex or triplex
formation between the probe and any nucleic acid in
the sample.

5

The probes according to this aspect of the invention
may be anchored to a solid support. Preferably, they
are present on an array so that multiple probes can
simultaneously hybridize to a single biological
10   sample. The probes can be spotted onto the array or
synthesised *in situ* on the array. (See Lockhart *et
al.*, Nature Biotechnology, vol. 14, December 1996
"Expression monitoring by hybridisation to high
density oligonucleotide arrays". A single array can
15   contain more than 100, 500 or even 1,000 different
probes in discrete locations.

The nucleic acid sequences, according to the invention
may be produced using such recombinant or synthetic
20   means, such as for example using PCR cloning
mechanisms which generally involve making a pair of
primers, which may be from approximately 10 to 50
nucleotides to a region of the gene which is desired
to be cloned, bringing the primers into contact with
25   mRNA, cDNA, or genomic DNA from a human cell,
performing a polymerase chain reaction under
conditions which bring about amplification of the
desired region, isolating the amplified region or
fragment and recovering the amplified DNA. Generally,
30   such techniques are well known in the art, such as
described in Sambrook *et al.* (Molecular Cloning: a
Laboratory Manual, 1989).

The nucleic acids or oligonucleotides according to the
35   invention may carry a revealing label. Suitable
labels include radioisotopes such as ^{32}P or ^{35}S, enzyme
labels or other protein labels such as biotin or

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fluorescent markers. Such labels may be added to the
nucleic acids or oligonucleotides of the invention and
may be detected using known techniques *per se*.

5   Antisense technology can be used to control gene
expression through triple-helix formation of antisense
DNA or RNA, both of which methods are based on binding
of a polynucleotide to DNA or RNA. For example, the
5' coding portion or the mature protein sequence,
10   which encodes for the protein of the present
invention, is used to design an antisense RNA
oligonucleotide of from 10 to 50 base pairs in length.
A DNA oligonucleotide is designed to be complementary
to a region of the gene involved in transcription
15   (triple-helix – see Lee *et al.* Nucl. Acids Res.,
6:3073 (1979); Cooney *et al.*, Science, 241:456 (1988);
and Dervan *et al.*, Science, 251: 1360 (1991), thereby
preventing transcription and the production of human
Tau or the protein capable of modulating Tau according
20   to the invention defined herein. The antisense RNA
oligonucleotide hybridises to the mRNA *in vivo* and
blocks translation of an mRNA molecule (antisense –
Okano, J. Neurochem., 56:560 (1991);
Oligodeoxynucleotides as Antisense Inhibitors of Gene
25   Expression, CRC Press, Boca Raton, FL (1988)).

Thus advantageously the expression of each of the
relevant proteins may be inhibited using antisense
technology which may be used to selectively confirm
30   the action of candidate compounds which may be
identified as potential treatments for Alzheimers or
other neurodegenerative diseases mediated by Tau
protein using the transgenic non-human animal
described herein, which expresses said human Tau
35   and/or said protein capable of modulating human Tau
protein.

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Recently it has become possible to manipulate the
expression of genes in animals by engineering genetic
switches in the genome of the animal which can be
designed to target expression or ablation of any gene
5   to any tissue at any defined time. (Inducible gene
targeting in mice using the Cre/lox system, a
companion to methods in enzymology 14, 381-392 (1998).
Using this technology expression of any of the
proteins according to the invention can be
10   manipulated, for example, such that expression only
occurs when the transgenic line has been established.
Accordingly, the vectors of the invention may include
a stop signal or sequence between the sequence capable
of directing expression of said human Tau or the
15   protein capable of modulating human Tau protein, which
stop signal is flanked by two loxP sites. When the
vector is used to establish the transgenic line as
described above and in the examples below, expression
of the relevant protein will not occur unless the Cre
20   recombinase protein is present. The Cre protein
catalyses reciprocal conservative DNA recombination
between the pairs of loxP sites with the resulting
excision of the stop sequence located between the loxP
sites. The Cre protein may itself be expressed in
25   another transgenic animal which is mated with the
first, to remove the stop sequence following the
reciprocal combination event between the two loxP
sites to switch on expression of the appropriate
sequence in the transgenic animal. This technique
30   also permits the DNA sequence encoding the proteins
according to the invention to be excised by the Cre
protein by including in the appropriate nucleic acid
vector loxP sites flanking the sequences encoding
human Tau and/or the protein capable of modulating
35   human Tau protein. Such vectors can be used to
investigate the role of null mutations or knock-outs
of the sequences encoding the proteins in the

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transgenic animal according to the invention.

In accordance with the present invention, a defined
nucleic acid includes not only the identical nucleic
5   acid but also any minor base variations including in
particular, substitutions in cases which result in
such as for example a synonymous codon (a different
codon specifying the same amino acid residue) due to
the degenerate code in conservative amino acid
10 substitutions. The term "nucleic acid sequence" also
includes the complementary sequence to any single
stranded sequence given regarding base variations.

A further aspect of the invention comprises a method
15 of making a transgenic non-human animal which
expresses a human Tau protein comprising the steps of:
(a) introducing into an embryo cell of said animal a
nucleic acid vector according to the invention; (b)
introducing the embryo from step (a) into a female
20 animal; (c) sustaining the female in step (b) until
such time as the embryo has sufficiently developed and
is borne from the female; and (d) sustaining the
transgenic animal.

25 A further method of generating a transgenic non-human
animal which expresses a human Tau protein comprises
the steps of (a) introducing sequentially or
simultaneously into an embryo cell of said animal a
nucleic acid vector comprising a transgene encoding
30 said human Tau protein; and a nucleic acid vector
comprising a sequence of nucleotides which upon
integration into the genome of said animal are capable
of preventing expression of endogenous Tau protein
from said animal; (b) introducing the embryo from step
35 (a) into a female animal; (c) sustaining the female in
step (b) until such time as the embryo has
sufficiently developed and is borne from the female;

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and (d) sustaining the transgenic animal.

Another method of generating a transgenic non-human
animal which is a model for diseases such as
Alzheimers disease, comprises crossing a first
transgenic non-human animal expressing human Tau
protein from a vector according to the invention with
a second transgenic non-human animal expressing a
protein capable of modulating human Tau protein
according to the invention, selecting among the
progeny those that carry both expression of said human
Tau protein and said protein capable of modulating
human Tau protein.

As described above, the Cre/lox technology can be used
to manipulate expression of the proteins in each of
the transgenic non-human animals described herein by
incorporation of loxP sites flanking an appropriate
DNA sequence. The sequence may be one or both of
those encoding either human Tau or the protein capable
of modulating human Tau protein themselves or
alternatively a stop sequence or codon which prevents
expression of the above proteins unless a
recombination event occurs in the presence of Cre
recombinase to remove the stop sequence. The vectors
used according to this aspect of the invention, to
generate the transgenic non-human animals, are
incapable of replication in yeast.

A further aspect of the invention comprises a
transgenic non-human animal that is a model for
Alzheimers disease or for another neurodegenerative
disease, which animal comprises an introduced DNA
sequence encoding and capable of expressing the
protein Tau in the nervous system of said animal and
also comprises a DNA sequence encoding and capable of
expressing a protein capable directly or indirectly of

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modulating the human Tau protein. In this aspect of
the invention the human Tau and the protein capable of
modulating human Tau are preferably those encoded by
the sequences on the vectors according to the
5   invention as described above.

A further aspect of the invention comprises a method
of generating a transgenic non-human animal which is a
model for Alzheimers disease or related
10   neurodegenerative disorders, comprising the steps of
crossing a first transgenic non-human animal
comprising a vector having, i) a nucleic acid sequence
encoding a human Tau protein, ii) a sequence capable
of directing expression of said human Tau protein in
15   the nervous system of said animal and iii) a targeting
sequence which facilitates integration of said vector
into the genome of said animal, with a second
transgenic non-human animal comprising a vector
capable of expressing a protein capable of modulating
20   human Tau protein according to the invention,
selecting among the progeny those that express both
human Tau protein and said protein capable of
modulating human Tau protein.

25   Another transgenic non-human animal according to the
invention is also provided by crossing a first
transgenic non-human animal expressing human Tau
protein with another non-human animal transgenic for
the protein which modulates human Tau protein.
30   Therefore, according to this aspect of the invention
there is provided a method of generating a transgenic
non-human animal which is a model for Alzheimers
disease or related neurodegenerative disorders,
comprising the steps of crossing a first transgenic
35   non-human animal comprising a vector having, i) a
nucleic acid sequence encoding a human Tau protein,
ii) a sequence capable of directing expression of said

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human Tau protein in the nervous system of said animal
and iii) a targeting sequence which facilitates
integration of said vector into the genome of said
animal, with a second transgenic non-human animal

5   comprising a vector according to the invention,
selecting among the progeny those that express both
human Tau protein and said protein capable of
modulating Tau protein.

10   The term "progeny" or "offspring" is intended to
include the resulting product of a mating between the
transgenic animals described provided it carries a
vector according to the invention. Also included are
germ cells from said transgenic animals which may

15   themselves be used to produce further offspring
comprising a vector according to the invention stably
integrated into its genome.

Preferably, the non-human animal used in accordance

20   with the methods of the invention is a mammal and even
more preferably a mouse.

The nucleic acid vectors described can be introduced
into the embryonic stem cells, by for example

25   electroporation. Microinjection of the cells is
performed on the embryo when it is at the one cell
stage, thus ensuring that the nucleic acid vector will
be incorporated into the germ line of the animal and
thus be expressed in all cells of the animals for

30   subsequent transmission to progeny. A further aspect
of the invention comprises progeny of the transgenic
animal according to the invention, which progeny
carries any of the nucleic acid vectors according to
the invention stably integrated into their genome.

35

The transgenic animal may advantageously exhibit the
symptoms of Alzheimer's or other related

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neurodegenerative disorders mediated by human Tau
protein phosphorylation making it a suitable model for
the disease in humans. Compounds which modulate and
interfere with (either by enhancing or inhibiting) the
5   hyperphosphorylation of human Tau protein may be
identified by administering the compounds to the
animal. Compounds identified as enhancers may
advantageously be applied to the animal to enhance
development of the disease. Inhibitors of the disease
10   may be identified by monitoring the effects or the
phosphorylation profile of Tau protein in the animal
following application or administration of the
compound to the animal. The compounds may be
administered by any suitable route, such as orally or
15   intravenously.

Furthermore, the present invention provides a method
of producing a compound which modulates the human
kinase mediated hyperphosphorylation of human Tau
20   protein comprising the steps of any one of the above
described screening methods; and additionally:

(i)   synthesising the compound obtained or identified
in said method or a physiologically acceptable
25   analogue or derivative thereof in an amount
sufficient to provide said modulator in a
therapeutically effective amount to a patient;
and/or
(ii) combining the compound obtained or identified in
30   said method or an analogue or derivative thereof
with a pharmaceutically acceptable carrier.

The compounds isolated by the above methods also serve
as lead compounds for the development of analog
35   compounds. The analogs should have a stabilized
electronic configuration and molecular conformation
that allows key functional groups to be presented to

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the Tau protein or the kinase in substantially the
same way as the lead compound. In particular, the
analog compounds have spatial electronic properties
which are comparable to the binding region, but can be
5   smaller molecules than the lead compound, frequently
having a molecular weight below about 2 kD and
preferably below about 1 kD. Identification of analog
compounds can be performed through use of techniques
such as self-consistent field (SCF) analysis,
10   configuration interaction (CI) analysis, and normal
mode dynamics analysis. Computer programs for
implementing these techniques are available; e.g.,
Rein, Computer-Assisted Modeling of Receptor-Ligand
Interactions (Alan Liss, New York, 1989). Methods for
15   the preparation of chemical derivatives and analogues
are well known to those skilled in the art and are
described in, for example, Beilstein, Handbook of
Organic Chemistry, Springer edition New York Inc., 175
Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic
20   Synthesis, Wiley, New York, USA. Furthermore, said
derivatives and analogues can be tested for their
effects according to methods known in the art; see
also supra. Furthermore, peptidomimetics and/or
computer aided design of appropriate derivatives and
25   analogues can be used.

It will be appreciated that not every vector, which
may otherwise be referred to as a transgene, will
function optimally in every cell or animal type. Thus,
30   routine experimentation may be required to identify or
establish the best kinase or Tau isoform or promoter
sequence for any given cell or animal type.

Antibodies to the protein or polypeptide of the
35   present invention may, advantageously, be prepared by
techniques which are known in the art. For example,
polyclonal antibodies may be prepared by inoculating a

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host animal, such as a mouse, with the polypeptide
according to the invention or an epitope thereof and
recovering immune serum. Monoclonal antibodies may be
prepared according to known techniques such as
5   described by Kohler R. and Milstein C., Nature (1975)
256, 495-497. Advantageously, such antibodies may be
included in a kit for identifying the human Tau or the
kinase in a sample, together with means for contacting
the antibody with the sample.
10

The invention may be more clearly understood from the
following exemplary embodiment by reference to the
accompanying Figures wherein;

15   Figure 1:   is an illustration of the recombinant
DNA construct used to target the mouse
Tau locus. The triangles represent the
loxP sites. The black boxes indicate a
part of the exon 1 of the mouse Tau
20   gene. BSSK+ denotes the bluescript
cloning vector. Pgk-hyg represents the
hygromycin marker gene. The middle
figure shows a partial structure of the
wild-type mouse Tau gene. Nco 1 is the
25   unique site on exon 1 into which the
entire construct is introduced. The
lower figure shows the construct ready
for introduction into the ES cells and
if homologous recombination occurs in
30   the mouse genome, the different probes
used with different enzyme digestions.
Details are in the text under the
section.

35   Figure 2:   is an illustration of the Southern Blot
used to identify transgenic mice
incorporating the human Tau 40 cDNA at

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the embryonic stage. 5 of the 46 pups
injected at the embryonic stage
contained the DNA.

5   Figure 3:   is an illustration of a Western Blot
results indicating a 64 kDa Tau protein
in three different transgenic mouse
strains, and probed with antibodies HT-
7 and Tau-5.

10

Figures 4 & 5: are illustrations of the different
digestions using rare cutting
restriction enzymes in a restriction
map of the human Tau gene.

15

Figure 6:   is an illustration of the expression of
human GSK-3β in brain of transgenic
mice (A) and activity of human GSK-3β
in the brain of transgenic animals

20   using a synthetic substrate peptide
(B).

Figure 7:   is an illustration of the results of a
Western Blot of brain extracts of GSK-

25   3β[S9A]/htau40 double transgenic mice,
5 weeks old. Brain extracts from wild-
type (WT), GSK-3β[S9A] single
transgenic ([S9A]-5), htau40 single
transgenic and GSK-3β[S9A]/htau40

30   double transgenic mice were
immunoblotted with the specified
monoclonal antibodies. For Tau-5
immunodetection, 6 times less extract
was applied than for AT8 and AT-180

35   staining. Intense hyperphosphorylation
of human protein tau was evident by
reaction with monoclonal antibodies AT-

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8 and AT-180 in the double transgenic
animals of all three lines generated.
Relative Mr is indicated on the left in
kDa. The single and double accolades
on the right denote the endogenous
murine and the transgenic human protein
tau respectively.

Figure 8:   is an illustration of the method of
producing the loxP - hygromycin
construct. This construct is incapable
of replication and/or of expressing
exogenous proteins in yeast.

Figure 9:   is a restriction digest of the
construct of Figure 8 using various
restriction enzymes.

Figure 10:   illustrates a restriction map of the
construct of Figure 8.

Figure 11:   is an illustration of the results
obtained by probing a cell line to
ensure the presence of the constructs.

Figure 12:   is an illustration of Western Blotting
of brain extracts of GSK-3β[S9A]
transgenic mice of 7 months old. Each
panel compares brain extracts from 2
individual wild type (wt) mice and from
2 individual GSK-3β[S9A] transgenic
mice, all about 7 months old,
immunoblotted with antibodies Tau-5,
PHF-1, AT-8 and AT180 as indicated with
each panel. Brain homogenates were
purified from mouse IgG prior to
electrophoresis.

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Figure 13:   is an illustration of the effect of
               alkaline phosphatase pretreatment on
               hyperhosphoryled protein tau. Brain
               homogenates of single and double htau-
5   40-5 and GSK-3β[S9A]/htau40 transgenic
               mice were either applied untreated, or
               after incubation at 37°C for 3 hours
               without or with alkaline phosphatase
               (0.5 unites per μl) prior to Wester
10   Blotting. For staining with antibodies
               Tau-5 and Tau-1, the amounts of extract
               applied were 6 times less than for
               blotting with AT-8 and AT-180. Note
               the reduction in signals and the
15   increase in electrophoretic mobility as
               described and discussed in the text.

Figure 14:   (a) and (e) are graphic representations
               of the recombinant DNA constructs used
20   to generate transgenic mice that
               express a mutant form of GSK-3β,
               denoted GSK3-β[S9A] and htau40; (b) and
               (c) are illustrations of the results
               obtained from a Western Blot of brain
25   and spinal cord extracts from
               transgenic and wild type mice,
               illustrating expression of the
               transgene in the transgenic compared to
               the wild-type mice; (d) and (g) are
30   illustrations of immunohistochemical
               localisation of the transgenic proteins
               in neuronal cell bodies and processes
               in the cortex and hippocampus in
               addition to motor neurons in the
35   ventral horn of the spinal cord,
               expressing both the human GSK-3β[S9A]
               mutant and the human tau transgene.

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Figure 15:   is an illustration of the results
                  obtained from a Western Blot using
                  brain extracts of double tau-4R x GSK-
                  3β[S9A] transgenic mice of five weeks
5   old immunoblotted using antibodies, AT-
                  8, AT-180, AD-2 and 12E8.

Figure 16:   is an illustration of the results
                  obtained from binding experiments of
10   tau protein to re-assembled
                  microtubules extracted from mouse brain
                  and spinal cord derived from htau-4R x
                  GSK-3β double transgenic mice compared
                  with htau-4R littermates.

15
Figure 17:   is an illustration of results obtained
                  from Western Blots of human and murine
                  tau protein which remained unbound to
                  microtubules, using antibodies Tau-1,
20   AT-180 and AD-2. Further shown are the
                  results of quantitative analysis of the
                  unbound protein by densitometric
                  scanning and normalisation to the
                  reaction with antibody Tau-5.

25
Figure 18:   is an illustration of the results
                  obtained from a Western Blot to
                  demonstrate that AD-2 and 12E8 epitopes
                  are differentially present on the bound
30   and free protein tau in the microtubule
                  extracts.

Figure 19:   is an illustration of sections of
                  diseased axons showing accumulation of
35   synapthophysin-bearing vesicles in
                  human tau transgenic animals.

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Figure 20:   is an illustration of sections of brain
                and spinal cord of double tau-4R x GSK-
                3β transgenic mice showing a dramatic
                reduction in the number of dilated
5   axons and lack of muscle wasting in the
                quadriceps of htau 40-1 x GSK3β mice.

Figure 21:   is an illustration of the results
                obtained from evaluating the effect of
10   co-expression of GSK-3β on the motoric
                aspect of the phenotype in different
                tests in double htau 40 x GSK-3β
                transgenic mice, relative to htau 40-2,
                GSK-3β and Wild-type mice. (a) is the
15   result of the 'uprighting reflex', (b)
                a rodwalking test measuring the number
                of mice that dropped off the rod and
                (c) the time the mice remained on the
                rod (d) the forced swim test and (e)
20   the grid hang test.

Transgenic GSK-3β[S9A] mice

Five independent transgenic founders were generated
25   that contained the human GSK-3β[S9A] mutant kinase
     under the control of the mouse thy-1 gene promoter, in
     the FVB genetic background. All experiments were
     comparatively performed with heterozygous mice from
     lines GS-3β[S9A]-5 and -1 in which GSK-3β expression
30   was highest and which were concordant in all
     phenotypic aspects.

     The human GSK-3β protein was revealed by Western
     Blotting (Fig 6A) and was enzymatically active towards
35   a GS-1 synthetic peptide. In brain homogenates of
     transgenic ice, GSK-3β kinase activity was about
     doubled relative to the activity in wild-type mouse

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brain (Fig 6B). Immunohistochemically, the human
protein was localized in neuronal cell bodies and in
processes in the cortex and hippocampus conform to and
expected from the known expression pattern of the
5   adapted mouse thy1 gene construct used (Moechars et
al., 1996 and references therein).

Transgenic human tau40 mice

10   Five independent transgenic founders were generated
that contained the human tau40 cDNA, embedded in the
adapted mouse thy-1 gene promoter; similar to the
construct used above. Three founder lines, i.e.
htau40-1, -2 and -5 transmitted the transgene in a
15   mendelian pattern and were analyzed. Western Blotting
with the human specific phosphorylation-independent
monoclonal antibody HT-7 demonstrated highest
expression of human protein tau in lines htau40-1 and
htau40-2 (Fig 3A). Western blots of total protein tau
20   with the phosphorylation-independent monoclonal
antibody Tau-5 were quantified by densitometric
scanning to demonstrate that the ratio of transgenic
human to endogenous mouse protein tau was about 1.5,
1.6 and 0.5 respectively in the three transgenic lines
25   (Fig 3B). In the brain of human protein tau40
transgenic mice of 4 to 8 weeks old, the antibody HT-7
stained the pyramidal nerve cell bodies and their
processes in the hippocampus and the cortex (Fig 7C),
while strong labelling was also evident in cortical
30   layer V.

**Transgenic GSK-3β[S9A] and double [GSK-3ß x tau-4R]
mice**

35   The transgenic mice expressing the longest human tau-
4R isoform have been described and characterized

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(Spittaels et al, 1999).

The present inventors have generated transgenic mice
that express a mutant form of human GSK3ß, denoted
5   GSK-β[S9A] since the cDNA contained an alanine residue
in position 9, instead of the wild-type serine to
prevent inactivation by phosphorylation (Woodgett,
1990). The cDNA was incorporated in a recombinant DNA
construct based on the mouse thy-1 gene promoter (Fig
10   14a) and transgenic mice were generated by micro-
injection, in the FVB mouse strain (Moechars et al,
1996, 1999; Spittaels et al, 1999).

The human GSK-3β protein was demonstrated by Western
15   blotting in brain and spinal cord (Fig 14b). The
transgene was enzymatically active on a synthetic
peptide substrate, resulting in a doubling of the
total GSK-3β kinase activity in GSK-3ß mouse brain
homogenates, relative to wild-type mice (Fig 14c).
20   Immuno-histochemically, the human transgenic proteins
were localized in neuronal cell bodies and in
processes in the cortex and hippocampus (Fig 14d), as
expected for the adapted mouse thy1 gene construct
(Moechars et al., 1996, 1999; Spittaels et al, 1999).
25   In addition, motor neurons in the ventral horn of the
spinal cord also expressed the human GSK-3β [S9A]
mutant and the human tau transgene as well (Fig 14d,
g) (Spittaels et al, 1999). Both transgenes were thus
demonstrated to be expressed in the same neurons, in
30   the same regions of brain and spinal cord.

Double transgenic mice were obtained by cross-breeding
the single transgenic strains.

35

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Analysis of phosphorylation of protein tau

Extensive analysis was performed by Western Blotting
of mouse brain extracts with a battery of well-
5   characterised antibodies including antibodies specific
for different epitopes of protein tau known to be
phosphorylated by GSK-3β (Sperber et al., 1995). In
GSK-3β[S9A] transgenic mice, 4-8 weeks old, only minor
differences in electrophoretic migration was observed
10   relative to age-matched wild-type mice and analyzed by
Western Blotting with the PHF-1 antibody. The
hyperphosphorylation of murine protein tau in such
transgenic animals was evidenced by AT-180
immunoreactivity, but only by longer exposure of the
15   western blots. The additional, and wanted reaction
with antibody AT-8 remained, however, absent in the
single transgenic mice of less than 2 months old. In
the brain of older GSK-3β transgenic mice, i.e. aged
up to 16 months (Fig 12), the immunoreactivity of both
20   phosphorylation-dependent antibodies AT-8 and AT-180
were observed clearly, concomitant with a slower
electrophoretical mobility of the immuno-reactive
isoforms of protein tau detected also with monoclonal
antibodies Tau-5 and PHF1 (Fig 12).

25

Extensive immuno-histochemical analysis was performed
on both paraffin and on cyrostat sections cut from
brain of mice sacrificed at different ages and
processed and fixed following several different
30   procedures. Staining with the antibodies used in
Western Blotting and with many additional antibodies
failed to reveal appreciable and reproducible
phosphorylation of endogenous mouse protein tau in the
GSK-3β transgenic mice. It is obvious that human
35   protein tau is far better characterized than mouse
protein tau, and that the antibodies used are
primarily directed to human protein tau. Since,

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moreover, evidence for any tau-pathy is lacking in
mice, the present inventors decided to investigate the
role of GSK-3β in mediating phosphorylation of human
protein tau *in vivo*, by generating double transgenic

5   mice coexpressing human protein tau next to GSK-
3β[S9A] in the same neurons.

Definite hyper-phosphorylation of human protein tau
was demonstrated in brain extracts of these double

10   transgenic mice, even at the early age of 5 weeks, by
Western Blotting with antibodies AT-8, AT-180 and Tau-
5 (Fig 7). Brain of single transgenic littermates
expressing GSK-3β or human tau40 only, showed no or
much weaker AT-8 and AT-180 immunoreactivity and

15   contained no or much less of the slower migrating
isoforms of protein tau. By the same western blot-
methods, hyper-phosphorylation of human protein tau
was demonstrated in brain extracts of double tau4R x
GSK-3β [S9A] transgenic mice, even as young as 5

20   weeks. Western blotting with antibodies AT-8, AT-180,
AD-2 and 12E8 reacted with slow migrating human
protein tau isoforms in brain homogenates of double
transgenic mice, and these were virtually absent in
their single transgenic littermates (Fig 15).

25
Pre-treatment of brain extracts with alkaline
phosphatase prior to electrophoresis, yielded
identical protein tau patterns of all mice on the
Western Blots and abolished AT-8 and AT-180

30   immunoreactivity of both murine and human protein tau
.(Fig. 13). In addition, prior de-phosphorylation
increased immunoreactivity with antibody Tau-1 (Fig
13).

35

GSK-3β reduced the amount of protein tau bound to microtubules

We examined whether GSK-3β activity affects protein
tau binding to microtubules in brain and spinal cord
extracts. Cytoplasmic extracts, isolated in the
presence of taxol still can perform and assemble into
microtubular structures, despite the unfavourable
conditions of low tubulin concentrations, the presence
of proteases and other harsh conditions. Such re-
assembled preparations still allow association of MAP
with microtubules (Vallee 1982).

The binding of protein tau to re-assembled
microtubules extracted from mouse brain and spinal
cord, was significantly reduced in homogenates derived
from htau4R x GSK-3β double transgenic mice, compared
to htau4R littermates (Fig 16). The presence of
phosphatase inhibitors was essential while addition of
LiCl during the isolation procedure did not affect
this result, indicating that the tau phosphorylation
had occurred in vivo and reflected the condition as
was in the brain of the htau40-1x GSK-3β transgenic
mice (Fig 16).

Reduced binding to microtubules was evidently related
to hyperphosphorylation as demonstrated by analysis of
unbound protein tau, human and murine, that remained
in the supernatant of these extracts. This soluble
protein tau was hyper-phosphorylated as evidenced by
reaction with antibodies Tau-1, AT-180 and AD-2 (Fig
17). Quantitatively most reactive was the epitope
recognized by AD-2, corroborated by reaction with PHF-
1 antibody that recognizes the same phosphorylated
epitope on protein tau.

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Quantitative analysis by densitometric scanning and
normalization to the reaction with antibody Tau-5,
demonstrated an almost 4-fold increase of
phosphorylation at the epitope defined by these
5   monoclonal antibodies (Fig 17). Antibodies AT-180 and
Tau-1 revealed only a moderate increase in the
phosphorylation of their respective epitopes in
protein tau in the double transgenic mice, relative to
the single transgenic tau4R mice.
10

Next, we demonstrated that the AD-2 and 12E8 epitopes
are differentially present on the bound and free
protein tau in the microtubule extracts. Western
blotting revealed that the 12E8 epitope was detectable
15   on bound and free protein tau, as opposed to the AD-2
phospho-epitope which was not detectable in the
microtubule-associated protein tau (Fig 18).

The epitopes of AD-2 and 12E8 encompass Ser^{396}/Ser^{404}
20   (Buée-Scherrer et al., 1996) and Ser^{262}/Ser^{356} (Seubert
et al., 1995), respectively, and have been discussed
as pivotal in the tau-microtubule interaction, subject
to regulation by phosphorylation (Bramblett et al.,
1993; Sengupta et al., 1998). Our results suggest that
25   phosphorylation of the AD-2 epitope, in our conditions
by GSK-3ß, could indeed be essential for this
interaction.

Co-expression of GSK-3β reduced the axonopaty in CNS
30   **of htau40 transgenic mice**

A pathological hallmark of the tau4R transgenic mice,
i.e. the presence of dilated axons (Spittaels et al.,
1999) is now demonstrated to contain synapthophysin-
35   bearing vesicles, normally rapidly transported to the
synapse by the motor protein kinesin, which also

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accumulated in the diseased axons (Fig 19).
Consequently, the excess protein tau appeared to
inhibit axonal transport by binding to the
microtubules in the tau4R transgenic mice, and this
5   then caused the axonal dilatations and the axonopathy
(Spittaels et al, 1999).

In the brain and spinal cord of double tau4R x GSK3ß
transgenic mice, the number of dilated axons was
10   dramatically reduced (Fig 20, Table 1). The same
result was observed in double transgenic mice obtained
with a different parental trau transgenic strain
(Spittaels et al, 1999) and denoted as htau40-2 x GSK-
3ß (Table 1). Concomitantly, the grouping of atrophic
15   fibers and the fascicular atrophy in htau40 transgenic
mice, diagnostic for their neurogenic atrophy, was
dramatically reduced in the double transgenic mice.
The quadriceps of htau40-1 x GSK-3β mice was devoid of
any muscle wasting that is a pathological hallmark in
20   the htau40 animals (Fig 20) (Spittaels et al,1999).

**Co-expression of GSK-3β rescued the motorical
impairment of htau40 transgenic mice**

25   The axonopathy and the severe motor problems were both
directly correlated to the level of expression of the
human tau4R transgene protein, in 3 different htau4R
transgenic founder strains (Spittaels et al., 1999).
The effect of co-expression of GSK-3β on the motoric
30   aspect of the phenotype, was evaluated by five
different tests, in double htau40 x GSK-3β transgenic
mice, relative to htau40-1, GSK-3β and wild-type mice
(Fig 21).

35   Overall, the double tau4R x GSK-3ß transgenic mice
behaved in all tests significantly better than their

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single parental strains, with the exception of one
parameter in the rod-walking test (Fig 21).
Interestingly, the tests revealed important
characteristics of the single transgenic mice as well.

5

In the "uprighting reflex", the time needed to return
when forced to lay on their back (Fig 21a), the
evident impairment of the single tau-4R transgenic
mice was nearly completely corrected in the double

10   htau40 x GSK-3β transgenic mice (Fig 21a).

In the forced swimtest (Fig 21d) and in the grid-hang
test (Fig 21e) the double transgenic mice performed as
good as wild-type mice and significantly better than

15   single htau40 and single GSK-3ß mice (Fig 21b, e).

Most difficult to interpret was the rod-walking test
in which two independent parameters were measured: the
number of mice that dropped of the rod (Fig 21b) and

20   the time they remained on the rod (Fig 21c). The
first parameter clearly did not differentiate between
the single tau4R and double transgenic mice,
demonstrating that all the tau-4R transgenic mice were
unable to remaining on the rod, a characteristic not

25   affected by GSK-3ß co-expression. On the other hand,
the time that the mice remained on the rod was
restored to that of the wild-type mice in the double
transgenic mice, as opposed to the markedly reduced
time in the single tau4R transgenic mice (Fig 21c).

30   Since both parameters reflect different aspects of
behaviour, motoric capacity and ability, it will be
interesting to analyze this further by other means in
depth and with the additional effect of ageing.

35   Interestingly, although the GSK-3β transgenic mice as
such, displayed reduced motor ability in the three
psycho-motoric tasks relative to wild-type mice, the

double transgenic mice were always more successful
than the single htau40 mice. These observations
demonstrate that expression of GSK-3β already affected
the motoricity of the transgenic mice, but that its
co-expression with human protein tau rescued to a
large extend or even completely, the phenotype of the
tau-4R transgenic mice, as measured by endurance,
postural stability, motor coordination, equilibrium
maintenance and muscle strength.

Table 1. Quantification of dilated axons in brain and
spinal cord of transgenic mice

		Spinal cord		Cerebral cortex
Mouse type	n	Mean no. *	n	Mean no. **
WT	4	0	4	0
GSK	4	0	4	0
htau40-1 H	8	9.9	8	10.7
htau40-1 H x GSK	3	2.2	4	0.5
htau40-2 H	8	3.6	4	2.6
Htau40-2 H x GSK	4	0.3	4	0.3

Numbers of dilated axons in entire transversal sections of the
spinal cord (6 µm thick) and in coronal sections of the entire
right hemisperic cortex (40 µm thick) are presented. Numbers of
dilated axons in the cerebral neocortex from sections through the
hippocampus were counted. Silver impregnation (9 sections per
mouse type counted) and SMI-32 immunostaining (8 sections per
mouse type counted) yielded similar results. Mice used were
three months of age.

The Kruskal-Wallis analysis revealed a significant decrease of
the number of axonal dilations in both the spinal cord and
hemispheric cortex of htau40 x GSK double transgenic mice
compared to htau40 single transgenic littermates. (*) htau40-1 H
- htau40-1 H x GSK: p=0.0181; htau40-2 H - htau40-2 H x GSK:
p=0.0073. (**) htau40-1 H - htau40-1 H x GSK: p=0.0063; htau40-2
H - htau40-2 H x GSK: p=0.017.
WT denotes wild-type mice, n: number of mice analyzed.

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The hypothesis that GSK-3β is a major kinase capable of hyperphosphorylation of protein tau in brain was first approached and tested *in vivo*, by overexpression of a constitutively active human kinase, i.e. GSK-

5   3β[S9A] in the brain of transgenic mice using the mouse thyl gene promoter. The transgene was enzymatically active in brain and expressed mainly in hippocampal and cortical neurons, thereby about doubling the overall GSK-3β kinase activity. Murine

10   protein tau extracted from the brain of young GSK-3β[S9A] transgenic mice was somewhat hyperphosphorylated, as manifested by the presence of isoforms with slower electrophoretic migration, with some AT-180 immunoreactivity but weak or absent AT-8

15   reaction on western blots. In older mice tested at 7 and 16 months of age, endogenous protein tau isoforms with clearly retarded electrophoretic mobility and with strong AT-8 immuno-reactivity were evident in the brain. Isoforms of murine protein tau that migrated on

20   8% polyacrylamide gels as a broadened band, reacted with antibodies PHF-1 and Tau-5. The increased PHF-1 immunoreactivity caused by GSK-3β-mediated hyperphosphorylation can be attributed to phosphorylation of serine residues at positions 396

25   and/or 404 that participate in this epitope (Otvos et al. 1994). *In vitro* studies showed that hyperphosphorylation of these and other epitopes rendered the slower migrating tau isoforms.

30   The reasons to investigate not only endogenous mouse protein tau but also human protein tau as substrate for GSK-3β[S9A] in these transgenic mice, are many and not only practical. Evidently, all the typical and specific antibodies used to detect phosphorylated

35   epitopes on protein tau are directed against the human protein. In addition, evidence for any involvement of endogenous murine protein tau in tau-pathy is lacking.

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Therefore, the ability of GSK-β3 to mediate
phosphorylation of human protein tau was investigated
by generating double transgenic mice, i.e. mice that
coexpress the human tau40 protein isoform and the
5   human GSK-3β[S9A] mutant kinase. To this end,
transgenic mice were generated that overexpressed the
longest human protein tau isoform, i.e. human protein
tau40 containing 2 N-terminal inserts and 4
microtubule binding repeats. Using the same type of
10   gene promoter construct assured the expression of both
transgenes to coincide inside the same neurons in
brain. In the single and double transgenic mice,
human tau protein accounted for up to 60% of total
protein tau in the brain of the highest expressing
15   transgenic mouse line.

Immunodetection with HT-7 revealed a somatodendritic
localisation in addition to axonal staining, similar
to a previous report on human tau transgenic mice
20   (Götz et al. 1995), and resembling the localisation of
endogenous protein tau in central neurons (Tashiro et
al. 1997).

The cross-breeding yielded the expected numbers of
25   double transgenic mice offspring, which were
identified by genotyping and demonstrated by Western
Blotting to co-express human protein tau40 and human
GSK-3β[S9A]. In the brain of the double transgenic
mice, unambiguous and robust hyper-phosphorylation of
30   protein tau was evident as early as 5 week-old of age,
by the presence of slower migrating isoforms reacting
strongly with both antibodies AT-8 and AT-180. This
proved that the epitopes of antibody AT-8, involving
serine 199 and/or 202 residues and the epitope of
35   antibody AT-180, involving threonine 231, were
abundantly phosphorylated, not excluding additional
phosphorylation at other residues to induce the slower

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migrating tau proteins. Tau's binding to microtubules
was eliminated by the phosphorylation of several sites
(Mandelkow et al. 1995, Trinczek et al. 1995, Preuss
et al. 1997), among which residue Thr231 was of major

5   importance (Sengupta et al. 1998). De-phosphorylation
prior to electrophoresis destroyed both the AT-8 and
AT-180 immunoreactivity, increased the reaction with
antibody Tau-1 and increased the electrophoretic
mobility of protein tau.

10

The cDNA coding for human GSK-3β[S9A] (Sutherland et
al. 1993; Stambolic and Woodgett, 1994) was ligated in
the mouse thy1 gene (Moechars et al., 1996). A Pvul-
Notl restriction fragment was micro-injected into 0.5

15   day old FVB/N prenuclear mouse embryos. Transgenic
founders were identified by southern blotting of Stul-
restricted mouse tail-biopt DNA, hybridized with a
probe of 701 bp obtained by PCR with forward primer
5'CAAGGTCCCCGTTTCTCC3' and reverse primer

20   5'CAGGGGATAGTGGTGTGG3'. Routine genotyping of
transgenic offspring, bred into the FVB/N genetic
background, was performed on tail-biopt DNA with
forward primer 5'CCCCACCACAGAATCCA3' located in the
mouse thy1 gene and with reverse primer

25   5'GCTGCCGTCCTTGTCTCT3' located in the human GSK-3β
cDNA. Human Tau40 was ligated in the mouse thy1 gene.
A Pvul-Notl restriction fragment was micro-injected
and transgenic founders identified by southern
blotting of Stul-restricted mouse tail-biopt DNA. The

30   probe of 135 bp was obtained by PCR with forward
primer 5'CCCCACCACAGAATCCA3' located in the mouse thy1
gene and reverse primer 5'GCCCCCCTGATCTTTCC3' located
in the human tau40 cDNA. Routine genotyping of
transgenic offspring, bred into the FVB/N genetic

35   background, was performed on tail-biopt DNA by PCR
with a forward primer 5'CTGGGGCGGTCAATAAT3' located in
the human tau40 gene and a reverse primer

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5'CAAGGTCCCCGTTTCTCC3' located in the mouse thy1 gene, yielding a 213 bp amplicon.

GSK-3β[S9A] protein levels in brain extracts were
5   estimated by Western Blotting with monoclonal
antibodies TPK I/GSK-3β (0.1 μg/ml) and htau40 protein
levels with monoclonal antibodies HT-7 (0.5 μg/ml) and
Tau-5 (0.5 μg/ml). Kinase enzymatic activity was
measured on brain homogenates after
10   immunoprecipitation and fractionation by ion-exchange
FPLC (Mono S) (Pharmacia, Uppsala, Sweden) (Van Lint
et al. 1993).

For immunohistochemistry of brain, mice were
15   anesthetized with nembutal and intracardially perfused
with either paraformaldehyde (4% v/v) or methacarn
(MC) (50% methanol, 30% chloroform, 10% acetic acid).
Brains were immersion-fixed overnight, dehydrated and
embedded in paraffin (unless stated otherwise).
20   Microtome sections (6 μm) were dewaxed, hydrated and
incubated with blocking solution, i.e. 3% BSA, 10%
normal goat serum in Tris Buffered Saline (TBS) (50 mM
Tris, pH7.4, 0.15 M NaCl). Incubation was for 12
hours with primary antibodies and for 1 hr with biotin
25   conjugated secondary antiserum in blocking solution,
and immunoreactivity was intensified with the Strep-
ABComplex/HRP system (Dako A/S, Denmark). A
monoclonal antibody to MAP2 (1/400) was used to mark
dendrites.
30
For immunohistochemical detection of human tau in the
htau40 transgenic mice, paraformaldehyde (4% in PBS)
fixed free-floating vibratome slices (40 μm) were
subsequently incubated with 300 μl blocking solution
35   (see above) for one hour and overnight with 250-300 μl
primary antibody (HT-7, 2.5 μg/ml; AT-8, 2.5 μg/ml;
AT-8, 2.5 μg/ml; PHF-1, 1/50) in blocking solution in

a 24 well Costar cell culture plate. Next, brain
sections were rinsed with 500 μl TBS (3x5'), incubated
with biotin conjugated secondary antibody (1/1000) for
one hour, washed (3x5') and pretreated with 500 μl

5   0.05 M Tris-HCl for 5 minutes. These tissue sections
were submerged in 300 μl Strept-ABComplex/HRP (1
droplet of both solutions per 15 ml 0.05 ml 0.05 M
Tris-HCl) for half an hour and successively washed
(3x5'), pretreated with 500 μl 0.05 M Tris-HCl for 5'

10   and stained with DAB.

For Western Blotting, brain tissue was homogenized in
2 ml of MES buffer with inhibitors, i.e. 0.1 M MES (pH
6.4), 0.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5

15   μg/ml leupeptin, 5 μg/ml pepstain, 1 μM okadaic acid,
200 μM PMSF, 20 mM NaF, 200 μM sodium orthovanadate, 5
μg/ml soybean trypsin inhibitor, 1% Triton-X-100, 1%
sodium desoxycholate and 0.1% SDS. After
centrifugation (100,000 g for 30' at 4°C), portions of

20   the supernatant were denatured and reduced prior to
separation on Tris-glycine buffered polyacrylamide
gels (8% SDS-PAGE) (Novex, San Diego, CA) and
transferred to nitrocellulose filters. Following
antibodies were used: monoclonal AT-8 and AT-180 (1

25   μg/ml), PHF1 (1/25) and Tau-5 (1 μg/ml). Signals were
quantified by densitometry and normalized to signals
obtained on the same blots with phosphate-independent
antibody Tau-5.

30   Since mouse immunoglobulins interfere with the AT-8
and AT-180 immunoreactivity on Western Blotting
(~50kDa), brain homogenates of GSK-3β transgenic mice
were incubated with immobilized protein-G (Pierce,
Illinois, USA) at 4°C for 2.5 hours and purified from

35   mouse IgG by centrifugation (8000 rpm, 5', 4°C). The
supernatant was denatured and reduced prior
electrophoretical separation.

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To dephosphorylate the tau protein, brain homogenates
were diluted in a dephosphorylation buffer (Boehringer
Mannheim) containing alkaline phosphatase (Boehringer
Mannheim, 0.5 unit/ μl homogenate) and gently stirred
5   at 37°C for 3 hours. Samples to be loaded on the gel
were prepared as mentioned above.

Antibodies HT-7 (directed to human tau), AT-8
(directed to phosphorylated Ser199 and/or Ser202
10   (Biernat et al. 1992) and AT-180 (directed to
phosphorylated Thr231 (Goedert et al. 1994) are
purchased from Innogenetics, Gent, Belgium. Anti-
TPKI/ GSK-3β was bought from Affinity, Nottingham, UK;
Tau-5 (recognizing tau, phosphate-independent) from
15   Beckton Dickinson, San Diego, CA; Tau-1 (directed to
non-phosphorylated Ser199 and Ser202 (Biernat et al.
1992) from Boehringer Mannheim, Germany and biotin
conjugated secondary antiserum from Biorad Labs, CA.
PHF1 (directed to phosphorylated Ser396 and Ser404
20   (Otvos et al. 1994) was a gift of P. Davies.

**Synthesis of the construct to target the mouse Tau
locus**

25   A 1.9 kb Not I fragment encoding the 3' loxP and the
Hygromycin B phosphotransferase gene driven by the
phosphoglycerate kinase (PGK promoter) was first
cloned into the BamHI site of the pBluescript vector.
Secondly, a 8 kb Mlu IβAat II fragment containing the
30   human Tau cDNA coding for the longest isoform of human
adult Tau driven by the mouse Thy I promoter was cut
out together with a 5' loxP site from the pGEM lox
vector and subcloned into the SmaI site of the
Bluescript vector (referred to as the Thy-I Tau 40
35   construct). From this recombinant vector a 10 kb, Sal
I β Not I fragment was introduced into the unique Nco

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I site of the exon I of mouse Tau gene. Prior to
electroporation into the ES cells, this targeting
vector was linearised with Not I restriction enzyme
and gel purified. The yield of the targeting vector
5   was analysed both by gel electrophoresis and optical
density using an UV spectrophotometer, wherein the O.D
was measured at 260nm.

ES cell culture, selection and genotyping

10

The ES cell line E14 (Hooper et al., 1987) was
cultured on mitomycin-treated STO fibroblasts, in
Glasgow ME medium containing non-essential amino
acids, 20% (w/v) fetal calf serum, 0.1mM 2-
15   mercaptoethanol and 1mM sodium pyruvate. Trypsinized
ES cells (1.5-2 x 10^7) were resuspended in 500 μl of
culture medium and electroporated with 10 to 15 μg of
the linearised targeting DNA, using an electric pulser
(Biorad Labs.) at settings of 200 V and 960 μF in
20   electroporation cuvettes of 0.4 cm electrode distance.
The electroporated ES cells were seeded onto mitomycin
treated STO fibroblasts in 25 cm^2 flasks and 40 hours
later, the medium was replaced with medium containing
100 μg/ml of Hygromycin B. Hygromycin resistant
25   colonies were picked up 10 to 14 days later after
electroporation and further expanded for genotyping.

DNA was isolated from the selected ES cell lines and
10 μg was digested with the desired restriction enzyme
30   for 4 to 6 hours. The digests were separated by
electrophoresis at 2 V/cm mechanism for 14 hours on
0.7% agarose gels resulting in an overnight run. The
following day the gels were stained by Ethidium
bromide and photographed, processed for capillary
35   transfer to nylon membranes. After baking and pre-
hybridisation, the blot was hybridised with the
radiolabelled probes at a concentration of 2-5 x 10^6

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cpm/ml and kept overnight at 60°C. Hybridisation was
carried out in 6X SSC, 5X Denhardt's solution, 1% SDS,
0.1% heparine, 10% Dextran sulphate and 0.1% Salmon
Sperm DNA. Membranes were washed at 60°C for one hour
5   in 0.3X SSC, 0.5% SDS and placed for autoradiographic
exposure at 70°C.

Genotyping by Southern blotting

10   For Southern blotting 10μg of genomic DNA was digested
for 5 hrs at 37°C and separated by electrophoresis in
0.7% agarose gels. DNA was transferred by capillary
transfer to a nylon membrane with 10X SSC (1.5M sodium
chloride, 150mM sodium citrate, pH 7.2). The membrane
15   was baked for 2 hrs at 80°C, pre-hybridised for 6 hrs
at 60°C in 6X SSC, 4X Denhardt's solution, 1% SDS, 100
μg salmon sperm DNA, 10% dextran sulfate and 0.05%
heparin. Hybridisation was carried out overnight at
60°C in the same solution supplemented with 2-5 x
20   10^6cpm/ml of the indicated [^{32}P]-labelled DNA probe.
The membrane was washed in 0.3X SSPE supplemented with
0.5% SDS for 1 hr at 60°C before autoradiographic
exposure with intensifying screens at -70 C for 1-7
days.
25
Different probes were designed to genotype the ES cell
lines. The ThyI-Tau-40 probe as mentioned above,
Hygromycin probe (a gift from Lieve Umans, Lutgarde
Serneels and Anton Roebroek) and a 3' probe. The
30   latter was made by a BamHI-Kpn I restriction of a 13kb
EcorV-Hind III fragment harbouring exon 1 and the
intron between the exons 1 and 2 of the mouse Tau
gene cloned in the Bluescript vector (gift by
Hirokawa,1997) yielding an external probe. The 3'
35   external probe thus obtained was purified from the gel
and used for the first screening of the electroporated

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ES cells cultured on Hygromycin containing selection medium. Since this probe recognised a region outside the construct, it helped us to figure out whether homologous recombination had occurred or not.(Fig.1).
5   The ThyI probe used to check the 5' region of the construct is obtained by ApaI digestion of the Thy I DNA (Prof.Van Der Putten).

Genotyping by PCR

10

Genotyping for mouse thyI-Tau40 transgenic mice by duplex PCR using the following two sets of primers: (i) the P16 forward primer in the mouse thy1 gene promoter: 5'CCCCACCACAGAATCCA in combination with
15   NE199 reverse primer in the human Tau-40 cDNA, 5'GCCCCCCTGATCTTTCC3', yielding an amplicon of 135 bp; (ii) the NE200 forward primer in the human Tau-40 cDNA 5'CTGGGGCGGTCAATAAT3' combined with the P62 reverse primer located in the mouse thy-1 gene
20   5'CAAGGTCCCCGTTTCTCC3', producing a 213 bp amplicon. The PCR programme consisted of 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 15 secs.

25   **Western Blotting**

Brain tissues were homogenized in 2 ml of 0.1M MES Buffer pH 6.4, 0.5mM MgCl$_2$, 1mm EDTA, 1mM EGTA, 1mM DTT, 0.2mM PMSF, 20mM NaF, 0.2mM Na$_3$VO$_4$, 1μM okadaic
30   acid, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml soybean trypsin inhibitor, 1% sodium desoxycholate, 1% Triton-X-100 and 0.1% SDS (Genis et al., 1995, with minor modifications). The brain extract so obtained was denatured at 95°C for 10 min and separated on a 8%
35   SDS-PAGE. The proteins were then transferred to nitrocellulose membranes and after blocking, were

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probed with suitably diluted monoclonal and polyclonal
antibodies. Antibodies used were HT-7 monoclonal
antibody (BR-01, clone HT-7, Innogenetics) and Tau-5
monoclonal antibody (60101A, Pharmigen) both diluted
5   1:1000.

Results

Thy-1 Tau 40

10

Expression of the human Tau 40 cDNA in the brain of
transgenic mice was obtained using the Thy-1 promoter.
Ten injection sessions yielded 46 pups (from 450
injected FVB oocytes transferred into 17 F1
15   pseudopregnant females). Genotyping by Southern
blotting technique identified 5 out of the 46 pups as
the human-Tau 40 founders (Figure 2). Additional
genotyping using duplex PCR identified these five mice
as founders. The F1 offspring of the founder Thy-1
20   Tau 40/4 suffered from premature death and the
complete strain died out within 2 months. Founder
Thy-1 Tau 40/3 failed to reproduce normally and as a
result three founder lines survived and appear
"genetically" healthy. The Western results showed a
25   ±70 kDa Tau protein in all the transgenic lines
(Figure 3) representing the longest human Tau isoform.
The transgene expression of all the founder lines
reached comparable levels with this relation of Line 1
showing the highest expression, followed by Line 2
30   closely and then Line 5. The strain of Thy-1 Tau 40/5
is being bred into homozygous strains.

PAC2 human Tau gene clone

35   The purified PAC2 clone was characterised by the
analyses of restriction fragments separated by Pulse

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Field Gel Electrophoresis (PFGE) and identified by
Southern blotting using the probes generated by PCR
(see materials and methods). The results of the
different digestions using rare-cutting restriction
5   enzymes are shown on the restriction map of the human
Tau gene (Figure 4). The PAC2 clone as sized by PFGE
was around 200 kb and housed the entire human Tau
gene, confirmed by Southern blotting using different
probes that identified the 5', middle and 3' regions
10   of the gene. To mention a few of the enzymes that
were used to linearise the construct outside the Tau
gene, we found that restriction with Sall and NotI
enzymes gave 4 and 5 bands respectively, while Pmel
cleaved the gene twice and Cpol linearised the clone
15   (Figure 5). The double digests of the PAC2 DNA of
NotI and Sall with Cpol analysed on the PFGE
demonstrated that one of the fragments made by the
single digests of the NotI and Sall enzymes was
cleaved but the Southern blotting done demonstrated
20   that Cpol did not cleave any part of the human Tau
gene. These analyses helped us to conclude that the
PAC2 DNA could only be linearised by Cpol without
fragmentation of the human Tau gene.

25   The linearised DNA was then purified using Qiagen
columns and dialysis chambers [Millipore Purification
columns, Spectra PorCE Dispodialyzer of Spectrum] of
different pore sizes, of which we found the tip-20
column of QIAGEN the most efficient as it yielded DNA
30   with least shearing and with a low elution volume a
concentration of 1 ng/μl was obtained, (one of the
drawbacks of the dialyses membranes) which is required
for microinjection. Genotyping by PCR identified 2
out of 9 pups as the PAC2 human Tau gene founders.
35   Although the PCR did show us results yet no expression
was observed in these mice as studies by Western
Blotting.

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Knockin-Knockout targeted vector

The loxP-PGK-hygromycin construct was cut out of the
pGEM vector by NotI and ligated into the BamHI site of
5   the Bluescript vector. Transformation of DH5α cells
with this 4.7kb construct yielded two positive
colonies out of the 20 screened. Restriction analyses
with SalI and ScaI enzymes gave the expected bands
indicating that ligation occurred in the right
10   orientation which was confirmed by sequencing with the
T7 primer. The 8 kb Thy-1 human Tau 40 construct (see
materials and methods) with the loxP site was
subcloned into the SmaI site of the above bluescript
vector. After transformation 5 out of the 20 colonies
15   screened harboured the insert. Restriction analyses
using ApaI, EcorV and Xmn1 enzymes identified 2
colonies holding the Thy 1 Tau 40 insert in the
desired orientation, additionally confirmed by
sequencing with primers T7 and NE201 (sequence located
20   in the PGK). The next step of the synthesis of the
construct involved the introduction of the 10kb SalI –
NotI fragment into the unique NcoI site of the exon 1
of the mouse Tau gene. 11 colonies were screened, of
which 2 were found to contain the insert after
25   transformation. Sequencing using the primers NE201
(5'-GATGTGGAATGTGTGCGA-3'), NE260 (5'-
CGCCAGGAGTTTGACA-3') (sequence located in the 5'
region of mouse exon 1) and NE261 (5'-
CTCATTCCTCCCACTCAT-3') (sequence located in the 5' end
30   of the PGK-hygromycin construct) was done to confirm
the orientation expected and of the 2 colonies, only
one had the right orientation. Figure 8 gives an
overall view of the making of the construct.
Restriction analyses carried out using various enzymes
35   as shown below indicated the presence of the complete
construct in the right orientation (Figure 9). The

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sizes have been estimated with the help of the 1 kb
marker.

	Enzymes	Band sizes as seen in the insert with the right orientation
5		
	ApaI	~9.2kb, ~6.6kb, ~0.8kb
	BamHI	~0.2kb, ~0.4kb, ~0.7kb, ~0.9kb, 4.9kb, ~9.5kb
10	EcorI	~3.0kb, ~3.4kb, 0.9kb, ~8.0kb, 0.7kb
	KpnI	~6.5kb, ~7.0kb, ~2.9kb
	NdeI	~13.5kb, ~1.2kb, ~1.6kb
	NslI	~10.9kb, ~5.4kb
	SacII	~5.2kb, ~3.4kb, ~7.7kb
15	ScaI	~8.7kb, ~2.5kb, ~5.2kb
	SmaI	~3.4kb, ~8.1kb, ~4.4kb, 0.2kb
	XbaI	~8.7kb, ~2.4kb, ~3.4kb, ~0.6kb, ~1.2kb

Figure 10 shows a restriction map of the concluded
20   construct. This construct is incapable of replicating
and/or expressing the exogenous proteins in yeast.
This final construct was linearised with NotI and
purified on a tip-100 column (Qiagen) which finally
gave a concentration of 2.25 μg/μl of which 8 μl was
25   used for electroporation into ES cells. The ES cells
that survived the electroporation were grown on
Hygromycin selective medium and after a fortnight
well-grown 333 colonies had been picked up for
culturing. With the help of Southern blotting using
30   the external 3' probe for the first screening (as
mentioned in the materials and methods), we were able
to pick up 6 potential positive cell lines in the
first screening. After the second screening of these
6 colonies with the internal Hygromycin and ThyI Tau
35   40 probe we obtained one cell line that contained the
right targeted construct in it. Besides, the ThyI
probe used finally also confirmed the presence of the

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5' region of the construct in the positive cell line
(Figure 11) and the 5' BamHI fragment hybridising with
this probe measured the same number of base pairs as
the predicted BamHI-fragment if the construct was
5   homogonously recombined. The marker used in the blots
is a 1 kb marker.

Probes	Digestion of ES DNA	Band Size	Homologous Recombination	Type of Probe
3'	KpnI	11.2kb	HR$_1$	External probe
	8.4kb	No HR		
Hygromycin	KpnI	11.2kb	HR	Internal probe
	BamHI	7.9kb	HR	Internal probe
ThyI Tau 40	KpnI	7.6kb		Internal probe
ThyI	BamHI	8.9kb	HR	Internal probe

This first positive cell line was used for injection
into blastocysts while further screening has resulted
20   in five more potential cell lines. Uterine transfers
have so far given 20 pups from three female mice of
which 6 are chimeric.

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CLAIMS

1.   A nucleic acid vector comprising:
       (a)   a nucleic acid sequence encoding a human Tau
             protein;
       (b)   a sequence capable of directing expression
             of said human Tau protein in the nervous
             system of a non-human animal; and
       (c)   a targeting sequence which facilitates
             integration of said vector into the genome
             of said animal so as to prevent expression
             of equivalent Tau protein or a related or
             equivalent protein from said animal in
             favour of said human Tau protein.

2.   A vector according to claim 1 further comprising
a sequence encoding a reporter molecule.

3.   A vector according to claim 2 wherein said
reporter molecule comprises the hygromycin Pgk-hyg
marker gene sequence.

4.   A vector according to any of claims 1 to 3
wherein said sequence encoding human Tau is a cDNA
sequence.

5.   A vector according to claim 4 wherein said cDNA
sequence encodes a Tau 40 isoform.

6.   A vector according to any preceding claim wherein
said sequence capable of directing expression of said
human Tau protein is a mouse promoter.

7.   A vector according to claim 6 wherein said mouse
promoter is a Thy-1 promoter.

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8.   A vector according to claim 7 wherein said
targeting sequence comprises a nucleotide sequence
exhibiting a sufficient degree of homology with said
sequence encoding said equivalent Tau protein in said
animal or flanking sequences thereof, to facilitate
integration of said vector into the genome of said
animal by homologous recombination.

9.   A vector according to claim 8 wherein said
targeting sequence comprises a NcoI restriction site
corresponding to the unique NcoI restriction site of
exon1 of the mouse wild type genome.

10.   A vector according to any of claims 1 to 9
further comprising two loxP sites flanking either of
the sequences of step (a) and (b).

11.   A vector according to any of claims 1 to 9
further comprising a stop sequence capable of
preventing expression of said human Tau protein and
which sequence is flanked by two loxP sites capable of
undergoing reciprocal conservative DNA recombination
in the presence of Cre recombinase with the resulting
excision of said stop sequence.

12.   A nucleic acid vector comprising:
    (a)   a nucleic acid sequence encoding a human
        protein capable of modulating human Tau
        protein;
    (b)   a sequence capable of directing expression
        of said protein in the nervous system of
        said animal; and
    (c)   a targeting sequence capable of facilitating
        integration of said vector into the genome
        of said animal optionally at a position
        corresponding to a sequence in said animal
        encoding an equivalent of said human protein

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so as to prevent expression of said
equivalent sequence in favour of said human
protein capable of modulating human Tau
protein.

13.   A vector according to claim 12 wherein said human
protein is capable of phosphorylating a human Tau
protein.

14.   A vector according to claim 12 or 13 wherein said
human protein is GSK-3β kinase.

15.   A vector according to any of claims 12 to 14
wherein said nucleic acid sequence in step a) is a
cDNA sequence.

16.   A vector according to any of claims 12 to 15
wherein said sequence capable of directing expression
of said protein capable of modulating human Tau
protein is a mouse promoter.

17.   A vector according to claim 16 wherein said
promoter is a Thy-1 promoter.

18.   A vector according to any of claims 12 to 16
further comprising two loxP sites flanking either of
the sequences of step (a) and (b).

19.   A vector according to any of claims 12 to 17
further comprising a stop sequence capable of
preventing expression of said protein capable of
modulating human Tau protein, and which stop sequence
is flanked by two loxP sites capable of undergoing
reciprocal conservative DNA recombination in the
presence of Cre recombinase with the resulting
excision of the stop sequence.

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20. A host cell transformed, transfected or injected with a vector according to any one of the preceding claims.

5   21. A host cell according to claim 20 wherein the cell is a non-human animal cell.

22. A host cell according to claim 21 wherein said non-human animal cell is a non-human mammalian embryo

10   cell.

23. A host cell according to claim 22 wherein said cell is an embryonic stem cell.

15   24. A method of making a transgenic non-human animal comprising the steps of:

      (a) introducing into an embryo cell of said animal one or more of a nucleic acid vector according to any of claims 1 to 19;

20   (b) introducing the embryo from step (a) into a female animal;

      (c) sustaining the female in step (b) until such time as the embryo has sufficiently developed and is borne from the female; and

25   (d) sustaining the transgenic animal.

25. A method according to claim 24 wherein said vector is introduced firstly into an embryonic stem cell which is subsequently introduced into a

30   blastocyst of said animal.

26. A method according to claim 25 wherein both of the vectors encoding said human Tau and said protein capable of modulating human Tau according to claims 1

35   to 11 and 12 to 19 respectively are introduced into said stem cell.

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27.   A method according to any of claims 24 to 26 wherein said non-human animal is a mammal.

28.   A method according to claim 27 wherein said mammal is a mouse.

29.   A method according to claim 24 or 25, comprising the step of introducing a vector according to any of claims 1 to 11 into a first animal and a vector according to any of claims 12 to 19 into a second animal, crossing said first and second animals and selecting among the progeny those that express both said human Tau and said protein capable of modulating human Tau protein.

30.   A method of making a transgenic non-human animal, which expresses a human Tau protein comprising the steps of:

    (a)   introducing sequentially or simultaneously into an embryo cell of said animal a first nucleic acid vector comprising a transgene capable of expressing said human Tau protein in the nervous system of said animal and a second nucleic acid vector comprising a sequence of nucleotides which upon integration into the genome of said animal is capable of preventing expression of endogenous Tau protein from said animal;

    (b)   introducing the embryo from step (a) into a female animal,

    (c)   sustaining the female in step (b) until such time as the embryo has sufficiently developed and is borne from the female; and

    (d)   sustaining the transgenic animal.

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31. A method according to claim 30 wherein each of
said first and second nucleic acid vectors are
introduced in the same embryo cell.

32. A method according to claim 30 or 31 wherein said
transgenic non-human animal is a mammal.

33. A method according to claim 32 wherein said
mammal is a mouse.

34. A method according to any of claims 30 to 33
wherein said second nucleic acid vector comprises a
sequence of nucleotides comprising a region of
homology with a sequence encoding an equivalent Tau
protein in said animal or with a region flanking or
adjacent said sequence so as to facilitate integration
of said vector into the genome of said animal by
homologous recombination.

35. A method of generating a transgenic non-human
animal which is a model for Alzheimers disease or
related neurodegenerative disorders, comprising the
steps of crossing a first transgenic non-human animal
comprising a vector according to any of claims 1 to 11
in its genome with a second transgenic non-human
animal comprising a vector according to any of claims
12 to 19 in its genome selecting among the progeny
those that express both human Tau protein and said
kinase.

36. A method according to claim 35 wherein said
nucleic acid vector in said first transgenic animal
comprises a vector according to claim 10 or 11.

37. A method according to claim 36 wherein said
second transgenic animal comprises a vector according
to any of claims 12 to 19.

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38. A method according to claim 34 which further comprises introducing into said second animal a vector comprising a transgene encoding Cre recombinase.

5   39. A transgenic non-human animal obtainable according to the methods of any of claims 24 to 38.

40. A transgenic non-human animal that is a model for neurodegenerative disorders, comprising:
10   (a)   an introduced DNA sequence encoding and capable of expressing the protein Tau in the nervous system of the animal; and
            (b)   a DNA sequence encoding and capable of expressing a protein capable directly or
15   indirectly of modulating Tau protein.

41. A transgenic non-human animal according to claim 40 wherein said sequence in step (a) comprises a vector according to any of claims 1 to 11.
20
42. A transgenic non-human animal according to claim 40 wherein said sequence according to step (b) comprises a vector according to any of claims 12 to 19.
25
43. A method of identifying a compound which modulates human kinase mediated phosphorylation of human Tau protein which method comprises administering a test compound to a non-human animal according to any
30   of claims 39 to 42 expressing both said human Tau protein and said human kinase and monitoring the phosphorylation profile of said Tau protein compared to one of said transgenic animals which has not been administered with the compound.
35
44. A compound obtainable according to the method of claim 43.

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45.   A pharmaceutical composition comprising a compound according to claim 44 together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

46.   Use of a compound according to claim 44 in the manufacture of a medicament for the treatment of neurodegenerative disorders.

47.   Use according to claim 46, wherein said neurodegenerative disorders comprise any of FTDP-17 (Fronto-temporal dementia associated with Parkinson's disease), Cortico-basal degeneration, progressive supranuclear palsy, multiple system atrophy , Pick's disease, Dementia Pugilistica, Dementia with tangles only, dementia with tangles and calcification, Down syndrome, Myotonic dystrophy, Niemann Pick's disease type C, Parkinsonism-dementia complex of Guam, Postencephalic Parkinsonism, Prion diseases with tangles, subacute sclerosing panencephalitis.

48.   A method of treating neurodegenerative disorders mediated by phosphorylation of human Tau protein comprising administering to a patient a compound as defined in claim 44 or a composition according to claim 45.

49.   A method of generating a transgenic non-human animal which is a model for Alzheimers disease or related neurodegenerative disorders, comprising the steps of crossing a first transgenic non-human animal comprising a vector having, i) a nucleic acid sequence encoding a human Tau protein, ii) a sequence capable of directing expression of said human Tau protein in the nervous system of said animal and iii) a targeting sequence which facilitates integration of said vector into the genome of said animal, with a second

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transgenic non-human animal comprising a vector
according to claim 12, selecting among the progeny
those that express both human Tau protein and said
protein capable of modulating Tau protein.

50. A method according to claim 49 wherein said
vector in said first and/or said second transgenic
non-human animal comprises a stop sequence capable of
preventing expression of said human Tau protein or
said protein capable of modulating Tau protein which
sequence is flanked by two loxP sites capable of
undergoing reciprocal conservative DNA recombination
with the resulting excision of said stop sequence.

51. A transgenic non-human animal obtainable
according to the method of claim 49 or 50.

FIG. 1.
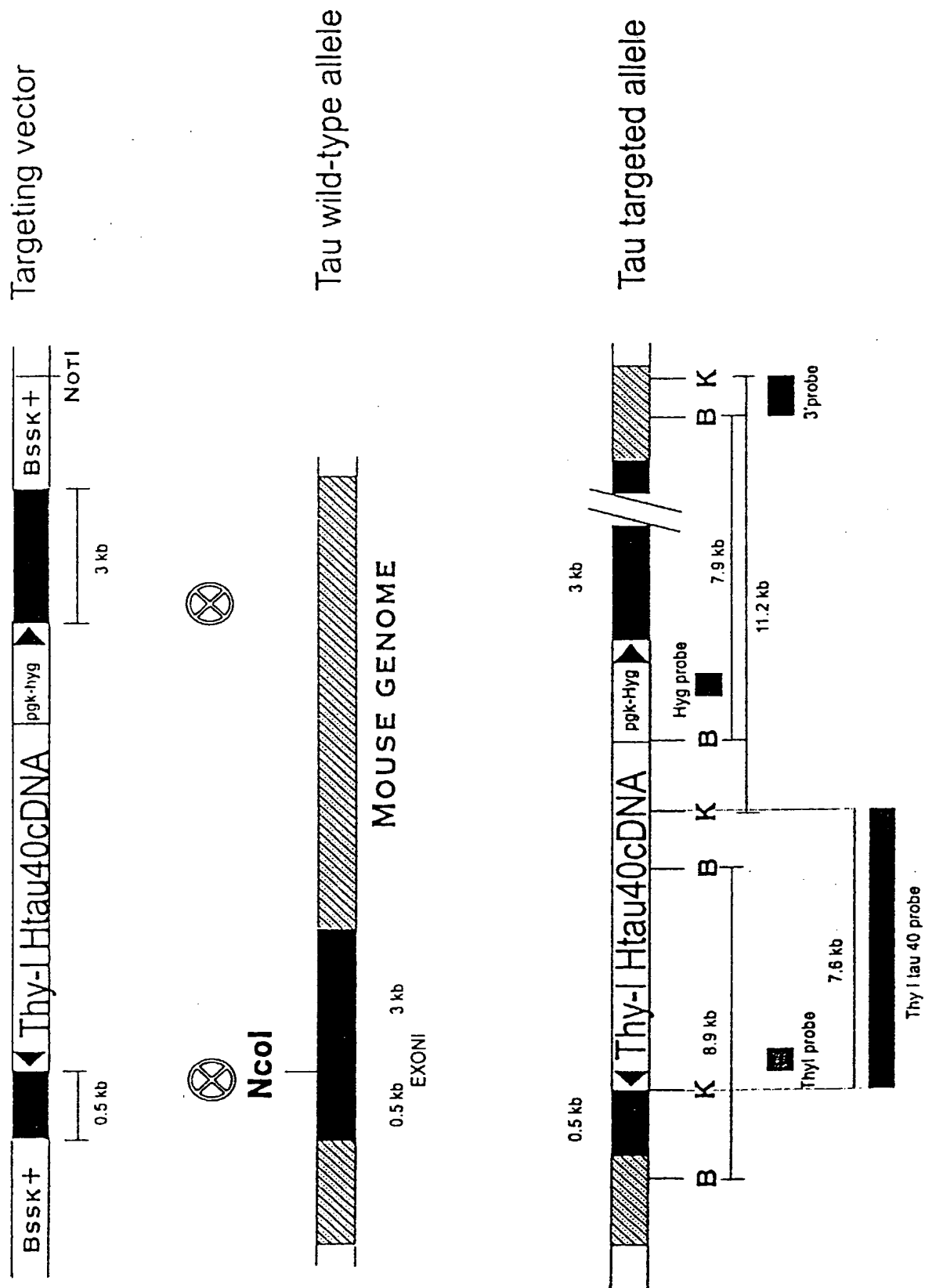
2/21

FIG. 2.
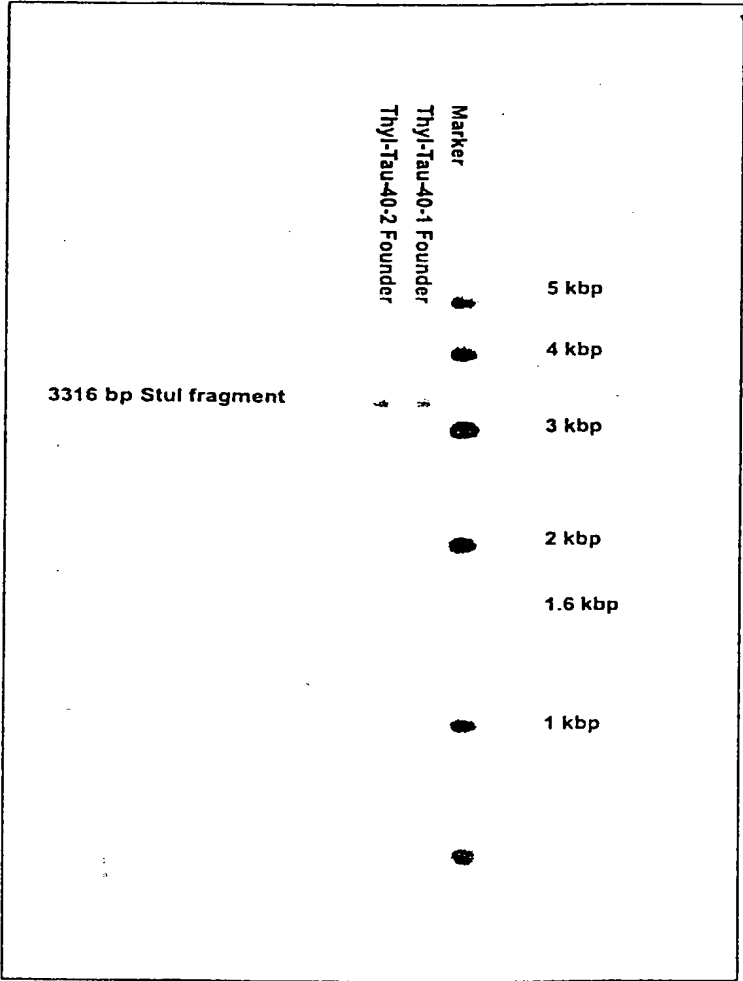
FIG. 3.
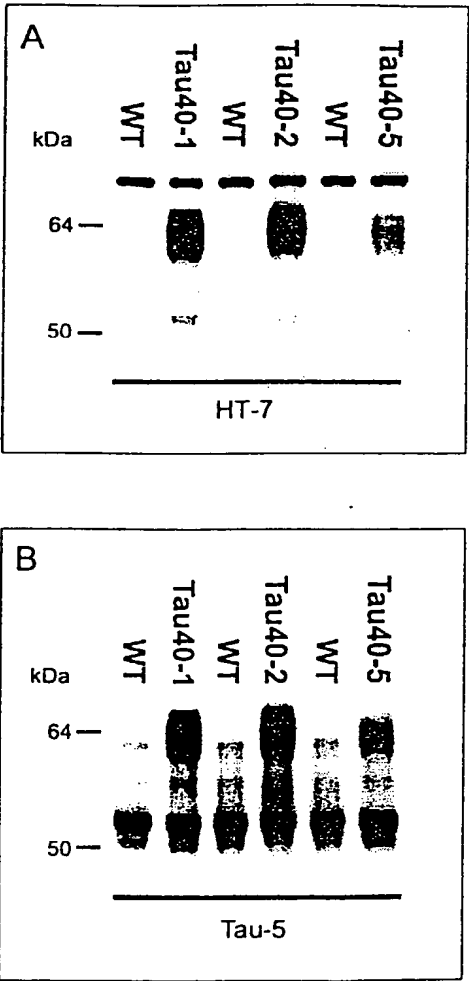
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FIG. 4.

FIG. 5.

The Human Tau gene

FIG. 6.

FIG. 7.

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FIG.8.

FIG. 9.

FIG. 10.

Map elements: BSSK | loxP | Thy1 tau 40 cDNA known seq 5886bp | pGK | Hygromycin | polyA | loxP | BSSK

880bp Thy1 unknown seq

1300bp Thy1 unknown seq

Restriction enzymes: Apa I, BamH I, EcoR I, Kpn I, Nde I, Nsi I, Sac II, Sca I, Sma I, Xba I

Apa I: 9.2 kb, 0.8 kb, 6.6 kb, 9.2 kb

BamH I: 9.5 kb, 0.2 kb, 0.7 kb, 0.4 kb, 0.9 kb, 4.9 kb, 9.5 kb

EcoR I: 3.4 kb, 7.0 kb, 8.1 kb, 0.7 kb, 0.9 kb, 3.0 kb

Kpn I: 2.9 kb, 6.5 kb

Nde I: 13.5 kb, 1.2 kb, 1.6 kb, 13.5 kb

Nsi I: 10.9 kb, 5.5 kb, 10.9 kb

Sac II: 7.7 kb, 5.2 kb, 3.4 kb, 7.7 kb

Sca I: 5.2 kb, 4.4 kb, 8.7 kb, 3.4 kb, 0.2 kb, 2.5 kb, 8.1 kb, 5.2 kb

Sma I: 4.4 kb

Xba I: 3.4 kb, 1.2 kb, 8.7 kb, 2.4 kb, 0.6 kb, 3.4 kb

FIG. 11.

| Kpnl - 3' probe | BamHI-Hygromycin probe | Kpnl-Hygromycin probe | BamHI-Thyl probe |

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FIG. 12.

12/21

FIG.13.

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FIG. 14.

(e)

(f)

(g)

FIG. 14.

(a)

1 kb

▬ Brain enhancers
▬ Thymus enhancers

(b)

anti-TPKI

brain

anti-TPKI

spinal cord

(c)

(d)

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FIG. 15.

FIG. 16.

FIG. 17

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FIG. 18.

human tau protein   mouse tau protein

FIG. 19.

FIG. 20.

FIG. 21.

(a) rugtest

(b) staaftest

(c) tijd tot afvallen

(d) zwemtest (2min)

(e) gridtest

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(72) Inventors; and

(75) Inventors/Applicants (for US only): GEERTS, Hugo, Alfons, Gabriel [BE/BE]; Janssen Pharmaceutica N.V., Turnhoutseweg 30, B-2340 Beerse (BE). SPITTAELS, Koenraad, Frederic, Florentina [BE/BE]; Center for Human Genetics, Campus Gasthuisberg, O & N 6, B-3000 Leuven (BE). VAN DEN HAUTE, Chris [BE/BE]; Center for Human Genetics, Campus Gasthuisberg, O & N 6, B-3000 Leuven (BE). VAN LEUVEN, Freddy, Kamiel [BE/BE]; Center for Human Genetics, Campus Gasthuisberg, O & N 6, B-3000 Leuven (BE).

(54) Title: DOUBLE TRANSGENIC ANIMALS AS MODELS FOR NEURODEGENERATIVE DISEASE
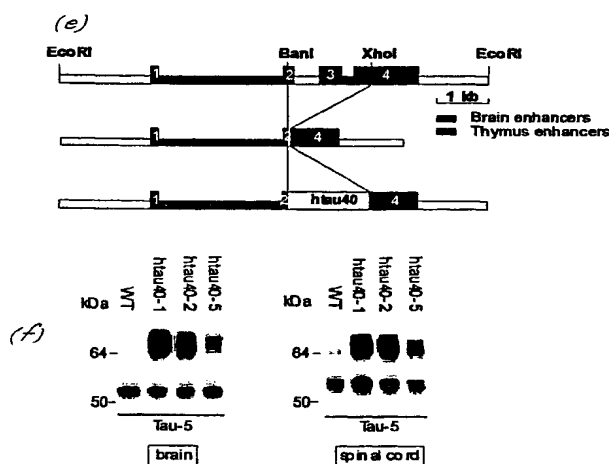
(57) Abstract: There is provided transgenic animals comprising: 1.a nucleic acid vector (a) sequence encoding a human Tau protein; (b) a sequence capable of directing expression of said human Tau protein in the nervous system of a non-human animal; and (c) a targeting sequence which facilitates integration of said vector into the genome of said animal so as to prevent expression of equivalent Tau protein; a nucleic acid vector comprising: (a) a nucleic acid sequence encoding a human protein capable of modulating human Tau protein; (b) a sequence capable of directing expression of said protein in the nervous system of said animal; and (c) a targeting sequence capable of facilitating integration of said vector into the genome of said animal optionally at a position corresponding to a sequence in said animal encoding an equivalent of said human protein.
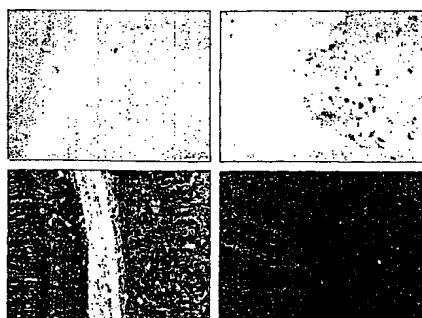
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional):* ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
20 December 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/00   A01K67/027   C07K14/47   C12N5/10   A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05466 A (INST OF PSYCHIATRY ;ANDERTON BRIAN HENRY (GB); MILLER CHRISTOPHER) 23 February 1995 (1995-02-23) page 4, line 1 – line 12 page 15, line 30 –page 16, line 6	40,43
Y	page 12, line 25; claims 12-15,20,30-38,45-49	1-51

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 2001	04/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Chambonnet, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GUO Q, SEBASTIAN L, SOPHER BL, MILLER MW, WARE CB, MARTIN GM, MATTSON MP.: "Increased vulnerability of hippocampal neurons from presenilin-1 mutant knock-in mice to amyloid beta-peptide toxicity: central roles of superoxide production and caspase activation." JOURNAL OF NEUROCHEMISTRY, vol. 72, no. 3, March 1999 (1999-03), pages 1019-1029, XP001020319 page 1020, column 1, paragraph 3 -column 2, paragraph 3	1-51
Y	MICHEL GOEDERT & MASATO HASEGAWA.: "The Tauopathies Toward an Experimental Animal Model" AMERICAN JOURNAL OF PATHOLOGY, vol. 154, no. 1, January 1999 (1999-01), pages 1-6, XP002923749 page 3, column 2, paragraph 2	1-11, 30-33
Y	GÖTZ, J. ET AL.: "Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform" EMBO JOURNAL, vol. 14, no. 7, 3 April 1995 (1995-04-03) - 1304, page 1313 XP002175363 EYNSHAM, OXFORD GB cited in the application the whole document	1-9, 30-33
Y	BRION J-P ET AL: "TRANSGENIC EXPRESSION OF THE SHORTEST HUMAN TAU AFFECTS ITS COMPARTMENTALIZATION AND ITS PHOSPHORYLATION AS IN THE PRETANGLE STAGE OF ALZHEIMER'S DISEASE" AMERICAN JOURNAL OF PATHOLOGY, PHILADELPHIA, PA, US, vol. 154, no. 1, January 1999 (1999-01), pages 255-270, XP000965124 ISSN: 0002-9440 the whole document	1-6, 30-33
A	KÜHN ET AL: "Advances in gene targeting methods" CURRENT OPINION IN IMMUNOLOGY, CURRENT BIOLOGY LTD. LONDON, GB, vol. 9, 1997, pages 183-188, XP002133099 ISSN: 0952-7915 the whole document	1-40

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2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SAUER BRIAN: "Inducible gene targeting in mice using the Cre/lox system." METHODS, vol. 14, April 1998 (1998-04), pages 381-392, XP001019712 figures 2-4	10,11,38
Y	BROWNLEES J ET AL: "TAU PHOSPHORYLATION IN TRANSGENIC MICE EXPRESSING GLYCOGEN SYNTHASE KINASE-3BETA TRANSGENES" NEUROREPORT,RAPID COMMUNICATIONS OF OXFORD, OXFORD,GB, vol. 8, no. 15, 20 October 1997 (1997-10-20), pages 3251-3255, XP000974561 ISSN: 0959-4965 cited in the application the whole document	12
Y	HARADA A. ET AL.: "Altered microtubule organization in small-calibre axons of mice lacking tau protein" NATURE., vol. 369, no. 6480, 9 June 1994 (1994-06-09), pages 488-491, XP002175364 MACMILLAN JOURNALS LTD. LONDON., GB ISSN: 0028-0836 the whole document	30-33
P,Y	SPITTAELS K ET AL: "PROMINENT AXONOPATHY IN THE BRAIN AND SPINAL CORD OF TRANSGENIC MICE OVEREXPRESSING FOUR-REPEAT HUMAN TAU PROTEIN" AMERICAN JOURNAL OF PATHOLOGY,PHILADELPHIA, PA,US, vol. 155, no. 6, December 1999 (1999-12), pages 2153-2165, XP000974569 ISSN: 0002-9440 the whole document	1-19
T	SPITTAELS K, ET AL.: "Glycogen synthase kinase-3beta phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice" J BIOL CHEM. , vol. 275, no. 52, 29 December 2000 (2000-12-29), pages 41340-41349, XP002175365 the whole document	12

-/--

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	DAWSON, H.N. ET AL.: "Tau distribution in a human tau gene transgenic/mouse tau knock out model" SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 25, no. 1-2, October 1999 (1999-10), page 790 XP001020341 abstract & 29th Annual Meeting of the Society for Neuroscience, Miami Beach, Florida, USA October 23-28 1999	30
T	DAWSON, H.N. ETAL.: "Inhibition of neuronal maturation in primary hippocampal neurons from tau deficient mice" JOURNAL OF CELLULAR BIOLOGY, vol. 114, no. pt 6, March 2001 (2001-03), pages 1179-1187, XP001020383 the whole document	1

2

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.:   44-48

Present claims 44 to 48 relate to a compound, a composition comprising it
and methods using thereof, defined by reference to a desirable
characteristic, namely   being obtainable by a method of screening using
a transgenic animal as described in claim 43.
The claims cover all compounds having this characteristic, all
compositions comprising them and all methods using thereof, whereas the
application provides no support within the meaning of Article 6 PCT and
no disclosure within the meaning of Article 5 PCTs. In the present case,
the claims so lack support, and the application so lacks disclosure, that
a meaningful search over the whole of the claimed scope is impossible.
Independent of the above reasoning, the claims also lack clarity (Article
6 PCT). An attempt is made to define the product by reference to a result
to be achieved. Again, this lack of clarity in the present case is such
as to render a meaningful search over the whole of the claimed scope
impossible. Consequently, the search has not been carried out for those
parts of the claims which appear to be not clear, not supported and
disclosed.

The applicant's attention is drawn to the fact that claims, or parts of
claims, relating to inventions in respect of which no international
search report has been established need not be the subject of an
international preliminary examination (Rule 66.1(e) PCT). The applicant
is advised that the EPO policy when acting as an International
Preliminary Examining Authority is normally not to carry out a
preliminary examination on matter which has not been searched. This is
the case irrespective of whether or not the claims are amended following
receipt of the search report or during any Chapter II procedure.

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			GB	2295395 A,B	29-05-1996
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